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Bevington, Sarah L.; Cauchy, Pierre; Cockerill, Peter N.

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Chromatin priming elements establish immunological memory in T cells without activating transcription

T cell memory is maintained by DNA elements which stably prime inducible genes without activating steady state transcription

Sarah L. Bevington, Pierre Cauchy and Peter N. Cockerill*

We have identified a simple epigenetic mechanism underlying the establishment and maintenance of immunological memory in T cells. By studying the transcriptional regulation of inducible genes we found that a single cycle of activation of inducible factors is sufficient to initiate stable binding of pre-existing transcription factors to thousands of newly activated distal regulatory elements within inducible genes. These events lead to the creation of islands of active chromatin encompassing nearby enhancers, thereby supporting the accelerated activation of inducible genes, without changing steady state levels of transcription in memory T cells. These studies also highlighted the need for more sophisticated definitions of gene regulatory elements. The chromatin priming elements defined here are distinct from classical enhancers because they function by maintaining chromatin accessibility rather than directly activating transcription. We propose that these priming elements are

members of a wider class of genomic elements that support correct developmentally regulated gene expression.

Keywords:

chromatin; enhancer; epigenetics; gene regulation; immunological memory; T cells transcription

Introduction

A defining hallmark of the mammalian immune system is the capacity to develop life-long immunity to infectious agents following episodes of infection. This ability is in large part due to the vast repertoire of antigen (Ag) receptors generated by V(D)J recombination in developing T and B lymphocytes. However, the immune system also needs to be tightly regulated to minimize inappropriate responses that would otherwise contribute to pro-inflammatory or auto-immune disorders. Consequently, naïve T cells, which have never before responded to foreign Ags, are very slow to react to their first encounter with the specific Ag recognized by their T cell receptors (TCRs). Naïve T cells are not “trigger happy,” and will only mount a full response when the foreign Ag is recognized in the right context on the surface of an Ag-presenting cell [1]. Up to this point, naïve T cells spend most of their life as small quiescent relatively inactive cells existing in what has been described by some as a “spore-like state” [2].

Once a response has been triggered, naïve T cells undergo a complex process of transformation over a 24 hour period to become highly active T blast cells capable of mounting very rapid responses to agents that activate TCR signaling (Fig. 1A). This process is accompanied by extensive BRG1-dependent

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Institute of Cancer and Genomic Sciences, Institute of Biomedical Research, University of Birmingham, Birmingham, West Midlands, UK

*Corresponding author:

Peter N. Cockerill
E-mail: p.n.cockerill@bham.ac.uk

Abbreviations:

Ag, antigen; ChIP, chromatin immunoprecipitation; DHS, DNase I Hypersensitive site; H3 K4me2, histone H3 Lysine 4 dimethylation; H3 K27Ac, histone H3 Lysine 27 acetylation; IL, Interleukin; LCR, locus control region; Pc, Polycomb; RNAPII, RNA Polymerase II; TF, transcription factor; TCR, T Cell Receptor; Trx, Trithorax.

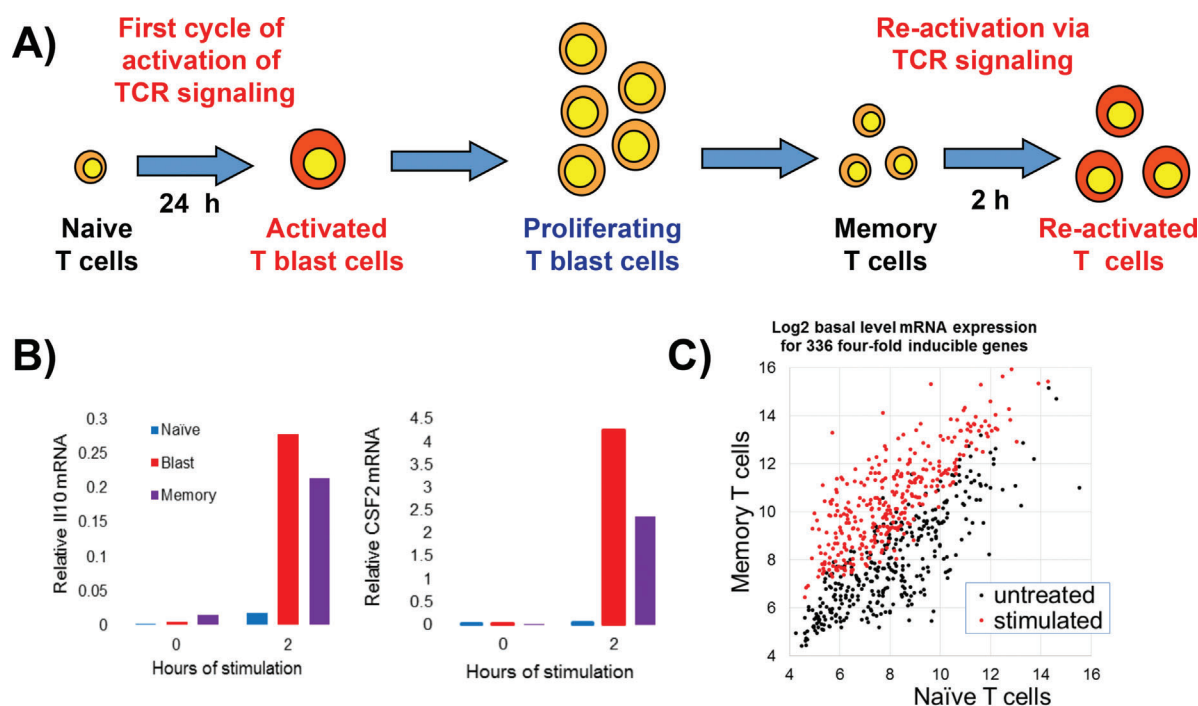


Figure 1. **A:** Stages of T cell activation that follow activation of TCR signaling in naïve T cells, which initially involves a slow transformation to rapidly proliferating and hyper-reactive T blast cells, some of which can later revert to memory T cells in vivo. **B:** Accelerated induction of *IL10* and *CSF2* mRNA expression in CD4+ previously activated T blast and memory T cells, compared to naïve T cells, in response to activation of TCR signaling pathways for 2 hours [13]. **C:** Plot of the log2 mRNA expression levels for the genes induced four-fold in untreated and stimulated memory T cells compared to naïve T cells [13].

chromatin remodeling [2, 3] during which the nuclei expand greatly in size and cells enter a period of rapid proliferation. In a natural setting, this initial transformation is followed by further differentiation into various subsets of specialized T cells, driven by environmental cues [1]. After an episode of infection has been resolved, the majority of responding T cells die. The remaining cells return to a quiescent state, and are maintained as memory T cells which are more sensitive to re-stimulation and which respond faster than naïve T cells [4–10].

Immune responses in T cells are largely driven by inducible genes which first gain the capacity to be efficiently activated by TCR signaling during the initial blast cell transformation process, a capacity which is retained by memory T cells [8, 10–13]. Recently activated T blast cells and long-term memory T cells each maintain a large proportion of immune response genes in a primed state ready for rapid re-activation. This principle is exemplified by human *CSF2*, encoding the GM-CSF gene, and *IL3* which we showed to be highly induced in recently activated CD4-positive T blast cells and memory T cells within just 2 hours, but is essentially not activated at all in naïve T cells under the same conditions (Fig. 1B) [13]. In parallel, we defined nearly 2,000 genes which are substantially more inducible in memory T cells than they are

in naïve T cells, and we identified potential mechanisms that might account for this difference [13].

T blast cell transformation primes inducible genes for rapid reactivation prior to terminal differentiation

Within most cell lineages, the defining events controlling differentiation to mature cells are associated with the activation of master regulator genes that switch on alternate gene expression programs. This is also the case for terminal differentiation of T cells to different classes of cells that mount varying responses to the infection according to environmental cues. For example, viral infections trigger a type one T helper cell response (Th1) mediated via activation of expression of the transcription factor (TF) TBX21/T-bet, whereas, parasite infections trigger a type two helper T cell response (Th2) mediated by activation of expression of the TF GATA3 [1, 14]. These two TFs function in immune responses to activate expression of alternate but overlapping subsets of genes [1, 14, 15]. However, no such mechanism has been defined that can account for the vastly different transcriptional responses of naïve T cells compared to recently activated T blast cells that have not yet undergone terminal differentiation, as illustrated in Fig. 1B. Indeed, immune response genes in previously activated T blast cells are fundamentally reprogrammed towards radically different responses without any substantial changes in the expression of TFs or other genes that might account for this effect. This fact was highlighted in a review article that noted a 95% overlap in the profiles of steady state transcription in naïve T cells compared to memory T cells [12]. In our own studies, we detected essentially no change in steady state transcription for the majority of the 2,000 genes

that were preferentially induced in memory T cells or for the genes where we found evidence for epigenetic priming in previously activated T blast cells [13]. These properties are depicted in Fig. 1C which shows data for 336 genes which are induced by at least four fold in T blast cells, but less than two fold in naïve T cells. Under steady state conditions, these genes are expressed at similar levels in T blast cells and naïve T cells.

In addition to chromatin priming [13], the enhanced responses of memory T cells are mediated in part by enhanced signaling responses. TCR engagement causes activation of Src family kinases, resulting in phosphorylation of the CD3 chains. Further, protein activation and phosphorylation events ultimately lead to calcium mobilization and activation of MAPK signaling pathways. While it is debated whether the threshold of TCR activation by antigen is lower for memory compared to naïve T cells [5, 7, 16–18], numerous studies have demonstrated that the two cell types have differences in the levels of activated signaling molecules downstream of the TCR [16, 17, 19]. Kersh et al. showed that, before the TCR is activated, the micro-domains within the plasma membrane of memory cells contain a higher concentration of the phosphoproteins required for signal transduction, such as phospho-LAT, and are therefore, more efficient at activating downstream pathways [16]. However, a difference in dependency of CD4 and CD8 memory T cells on certain signaling molecules, such as the TCR and SLP-76, suggests that this is not a unifying mechanism for all memory cells [20–22].

Polycomb and Trithorax-dependent mechanisms regulate a subset of T cell responses

Given the absence of significant changes within the TF network immediately following blast cell transformation, many investigators looked to epigenetic mechanisms as an explanation for the reprogramming of memory T cells, resulting in a large body of supporting evidence [11, 12]. Much of this effort has been directed at studying the opposing roles of developmental regulators of the Polycomb (Pc) and Trithorax (Trx) families of chromatin modifiers [23], as these factors are known to mediate transcriptional memory. The PRC1 and PRC2 Pc complexes can cooperate to both bind to and propagate the repressive histone H3 K27me3 modification within chromatin domains, making it possible for Pc to support the stable long term maintenance of the repressed state at silenced genes [23–25]. In contrast, the histone H3 K4me3 modification is inserted into actively transcribed promoter regions by the Trx group of SET domain proteins [23]. Previous studies investigating such chromatin modifications have led to the discovery of numerous immune response genes containing promoters that were repressed by Pc-dependent mechanisms in naïve T cells, and maintained in an active state by Trx-dependent histone H3 K4me3 modifications in effector and memory T cells [12, 26–31]. Conversely, other genes were shown to switch from an active conformation in naïve T cells to a repressed state in memory T cells. Loss of repression during the naïve to effector T cell transition was

seen at the GATA3, interferon γ , and interleukin-4 (IL-4) loci which switched from a high histone H3 K27me3 state to a high H3 K4me3 state [32, 33]. However, these observations could only account for a small subset of the thousands of genes which are activated much faster in memory T cells than in naïve T cells. For example, the human and mouse IL-3 and GM-CSF genes are expressed significantly higher in previously activated T cells than in naïve T cells, without any obvious involvement of Pc-dependent mechanisms in suppressing these loci in naïve T cells [13, 34]. There is also no evidence for repression of these genes by alternate mechanisms such as the repressive histone H3 K9me3 modification or DNA methylation [34]. It seems that these genes are not repressed, they are just off in naïve T cells.

The changing epigenetic landscape which is observed during T cell polarization is not confined to gene bodies and promoters. Other genome-wide studies have identified distal elements by mapping DNase I Hypersensitive sites, H3 K4me1 levels, and transcription factor binding in differentiated T cell subsets [35, 36]. Thousands of distal elements were identified in Th1 and Th2 cells, and although there was a distinct enhancer signature for each subset, over half of the elements were shared between the cell types [36]. These regions were described as active enhancers based on the binding of the histone acetyl transferase P300. However, because this is not a definitive measure of enhancer activity, the exact role of these elements in the context of gene regulation was at the time poorly understood.

Other previously defined mechanisms of transcriptional memory

It is by now well established that active chromatin modifications are retained at genes that exhibit transcriptional memory [37]. This allows certain genes to be re-activated either faster or more strongly next time they become stimulated by TCR signaling [11, 12, 28, 34, 38]. For example, genes that are primed in T cells typically have acetylated histones [38], but it has not always been clear how active modifications are either re-established after mitosis in dividing cells or maintained in the long term in quiescent cells. Transcriptional memory has also been previously associated with histone H3 K4me2, which is an active chromatin modification introduced by SET domain proteins, and similar to H3 K4me1 tends to be found at regions such as enhancers and not at actively transcribed promoters [39, 40]. Genes that are primed for reactivation in T cells and macrophages are known to be associated with this modification [34, 41, 42]. Hematopoietic genes that are poised for activation in hematopoietic progenitor cells carry this mark before the genes become active [43]. It is known that tissue-specific genes are marked by high intragenic H3 K4me2 levels, suggesting a global mechanism whereby priming with this mark not only reflects memory but cell fate, at least in T-cells [44]. The H3 K4me2 modification is also involved in a specialized form of transcriptional memory in organisms ranging from yeast to mammals whereby Set3 maintains genes in a transcriptionally poised but hypo-acetylated state [45].

The Set3/COMPASS complex binds to and maintains H3 K4me2 and helps to recruit poised RNA Polymerase II (RNAPII) to promoters [45]. However, this specific model cannot account for the many genes which are primed in T cells which are hyper-acetylated, not hypo-acetylated, and where phosphorylated forms of RNAPII are not retained [34].

The search for additional mechanisms controlling immunological memory

Despite the large body of data amassed from studies of terminally differentiated T cells and memory T cells, there remained no clear understanding as to why the majority of immune response genes were more active in these cells or how they became primed in the first place. Differentiation-driving TFs such as GATA3 and TBX21 will clearly contribute to priming of a subset of genes in memory T cells derived from differentiated cells, but are insufficient to account for the priming of thousands of genes during the initial blast transformation process. Our laboratory, therefore, embarked on a search for additional mechanisms that could explain the fundamental basis of how immunological memory is both established and maintained. To build a model consistent with the known facts it had to take into account (i) the lack of significant changes in steady state levels of transcription; (ii) the absence of universal memory T cell-specific TFs acting specifically at primed genes to globally maintain immunological priming, as opposed to differentiation; and (iii) the life-long maintenance of immunological priming in both dividing and quiescent cells. This meant that the immune system was most likely using the pre-existing TF network to maintain genes in a primed state, and that any changes in the transcriptional program would only be detectable when cells were re-activated. We also predicted that any stable mechanism for maintaining immunological memory would depend on stable binding of specific TFs that recruit chromatin modifiers to maintain chromatin in an active conformation. Therefore, We performed a screen for potential regulatory regions interacting with specific TFs that might make genes more responsive without influencing steady state transcription.

Inducible factors establish immunological memory by a hit-and-run mechanism

There are numerous previous studies in plants and animals that defined hit-and-run mechanisms which reprogram loci for transcriptional activation [37, 46–48]. We suspected that similar mechanisms might be at work in memory T cells and, therefore, used mouse T cell models to investigate this. We began by identifying all the open chromatin regions that exist as DNase I Hypersensitive Sites (DHSs) in naïve T cells, recently activated proliferating T blast cells, and in quiescent memory T cells via the now standard approach of performing genome-wide sequencing of DNA released from DHSs (DNase-Seq) (Fig. 2A and B) [13]. This was followed up by performing

genome-wide Chromatin Immunoprecipitation (ChIP-Seq) of activating histone modifications and candidate TFs (Fig. 2C). The DNase-Seq data are depicted in Fig. 2B as the profiles of ~17,000 DHSs centered within a 2kb window, and ranked from top to bottom in order of increasing DHS intensity in the T blast cells relative to the naïve T cells. Figure 2C shows the positions of specific DNA motifs for the TFs RUNX, ETS and AP-1, alongside ChIP-Seq analyzes showing that ETS-1 and JUNB bind to these regions, and also showing that H3 K4me2 is present in the adjacent chromatin. This integrated analysis revealed the existence of a specific subset of ~3,000 DHSs that were absent in naïve T cells but were stably maintained in both previously activated T blast cells and in memory T cells (Fig. 2B) [13]. We defined these 3,000 elements as primed DHSs (pDHSs). We also examined gene expression and mapped DHSs in each cell type following activation via chemical inducing agents (P/I) which directly activate TCR signaling pathways just upstream of the inducible TFs NFAT and AP-1. We found that the pDHSs were predominantly distal DHSs associated with genes that are preferentially induced in previously activated T cells (Fig. 3) [13]. Significantly, we found a strong correlation between the strength of induction of immune response genes and proximity of pDHSs to inducible enhancers regulating these genes. Based on these data, we established a model for immunological memory whereby pDHSs have a specialized function as “priming enhancers,” in contrast to the conventional enhancers which function in these loci to activate transcription. The same mechanisms of priming were observed in both CD4 and CD8 subsets of T cells, and are most likely universal throughout the T cell lineage [13].

The murine *Il10* (Fig. 3A) and human *IL3/CSF2* (Fig. 3B) loci provide good examples where pDHSs exist in close proximity to inducible enhancers that form inducible DHSs. These loci encompass several pDHSs which are bound by RUNX1 before the cells are stimulated, plus several inducible DHSs that recruit the inducible AP-1 family transcription factor JunB following activation. As stated above, these analyzes show an absence of the Pc-dependent modification at the pDHSs depicted here, but do point toward a repressive role for this modification within the transcribed coding region of the *Il10* locus in naïve T cells but not Th2 cells (Fig. 3A).

A defining feature of the pDHSs found in T blast cells is that they are highly enriched for binding sites for the constitutively expressed ETS and RUNX families of TFs (Fig. 2C) [13], and many of these motifs exist as the composite motif CAGGAAGTGGT which supports co-operative binding of ETS-1 and RUNX1 in T cells [49]. However, this feature alone is not sufficient to account for properties of pDHSs because RUNX1 and ETS-1 are constitutively expressed factors bound to many DHSs in both naïve T cells and T blast cells. An explanation for the redistribution of these pre-existing factors came from the frequent co-association of RUNX and ETS motifs with AP-1 motifs specifically in the pDHSs, and not in the other DHSs shared with naïve T cells (Fig. 2C). These data allowed us to postulate a hit-and-run mechanism whereby transient activation of AP-1 (and other TFs induced by TCR signaling), together with the pre-existing TFs, is sufficient to induce the opening of these DHSs during blast cell transformation (Fig. 4). In this model, the sustained TCR signaling induces AP-1,

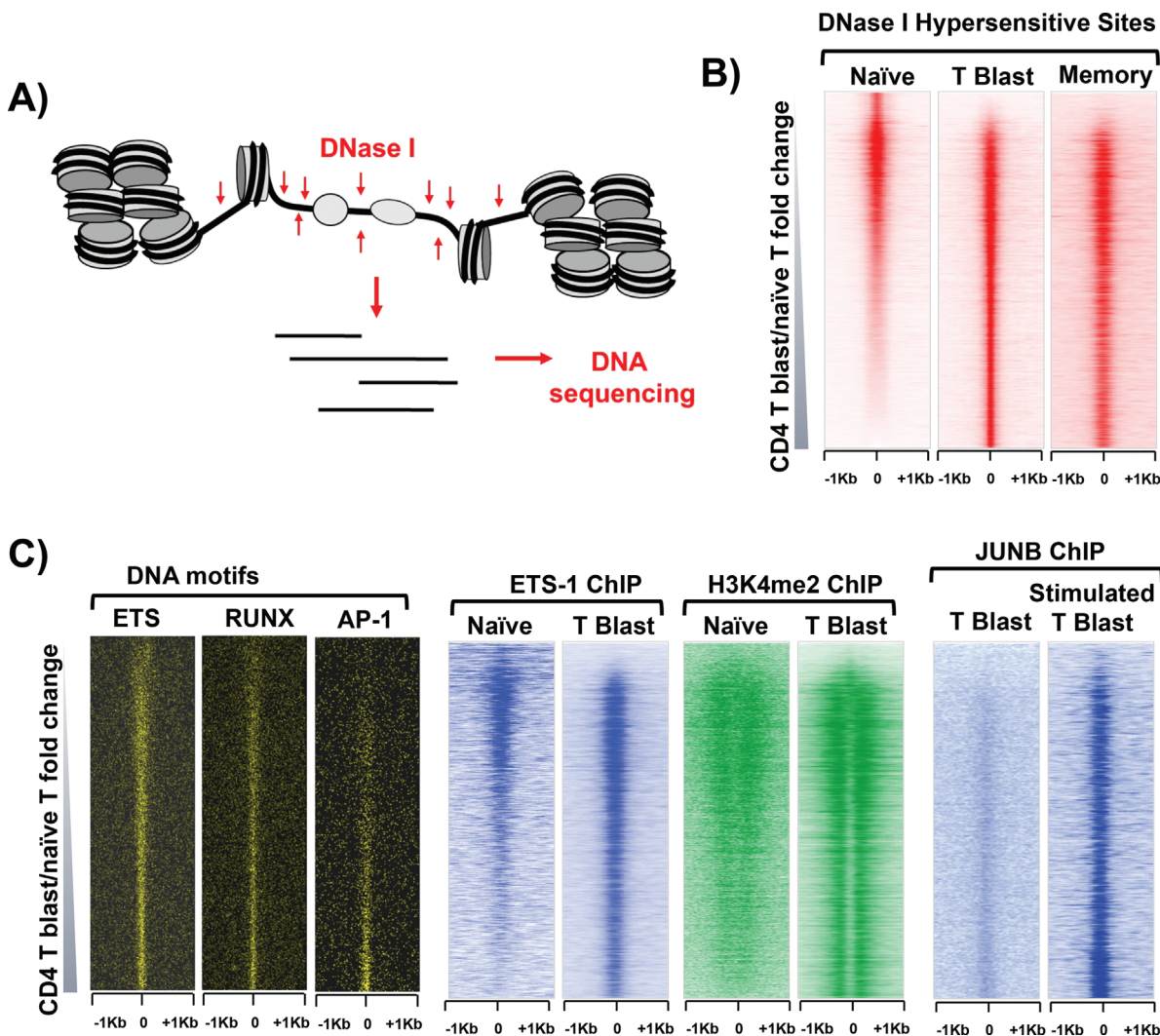


Figure 2. **A:** Outline of the method used for genome wide-identification of DHSs by DNase-Seq [79]. **B:** Genome-wide identification of DHSs enriched in both CD4+ T blast cells and memory T cells relative to naïve T cells [13]. Profiles are shown for the ~17,000 strongest DHSs present in naïve and/or T blast cells, centered on a 2 kb window and ranked in order of increasing intensity in T blast cells. **C:** The positions of predicted binding motifs for ETS, RUNX, and AP-1 TFs, and data from parallel ChIP-Seq analyses for the same DHSs as shown in B.

NFAT, and NF- κ B which cause substantial chromatin opening at both the inducible enhancers and the pDHSs. However, the inducible factors are only required for the initial recruitment of ETS-1 and RUNX1 to these newly accessible regions. Once formed, the constitutively expressed TFs are sufficient to maintain these pDHSs in the absence of the inducible TFs that helped create them. Therefore, it is the expression of the constitutive and inducible transcription factors at the same time which is required for the formation of the pDHSs.

The fact that pDHSs are created via a pathway that uses the pre-existing TF network, including TFs whose activity can be induced by signaling to activate specific regulatory elements, means that in principle no memory T cell specific factors are

needed to maintain immunological memory at most pDHSs. Remarkably, the TF motifs that are found in pDHSs are for the most part the same as the motifs that are found in inducible DHSs, with the possible exception of NFAT sites. They each engage with AP-1, ETS, and RUNX family TFs but nevertheless behave in fundamentally different ways. The key difference is not in the identity of the TFs but in the way they are used. The inducible DHSs tend to have on average two motifs for AP-1 and/or NFAT, but only one motif for ETS or RUNX; whereas, the converse is true for pDHSs [13]. The pDHSs rely on low-level binding of inducible TFs for their initial genesis, and use RUNX and ETS for maintenance; whereas, the inducible DHSs can only persist in the presence of inducible TFs.

Primed DHSs function at the level of chromatin accessibility

The specific functions of pDHSs as priming enhancers can most likely be explained by their ability to create active chromatin domains where there is greatly increased accessibility to the inducible factors that activate nearby inducible elements. For

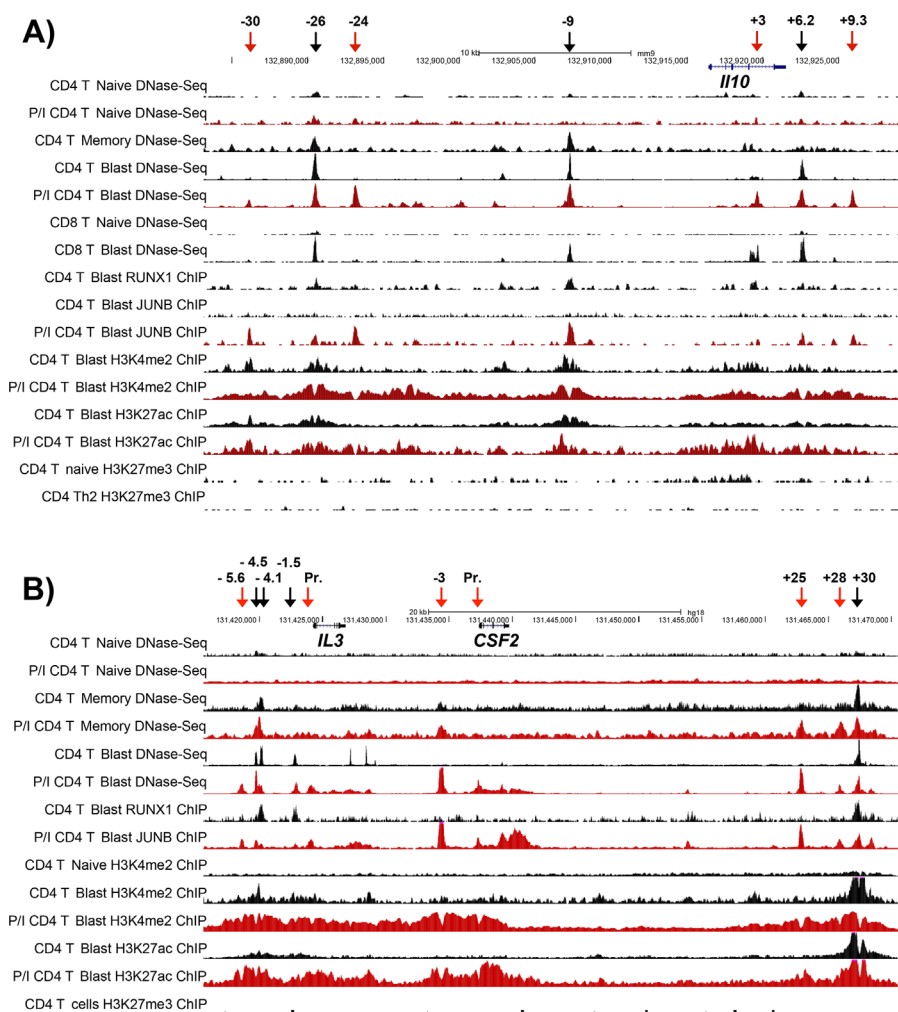


Figure 3. UCSC genome browser shots of the mouse *Il10* locus (**A**) and the human *IL3/CSF2* locus (**B**) showing DNase-Seq and ChIP-Seq in CD4 naïve T cells, CD4 memory T cells and CD4 T blast cells derived from C42 transgenic mice which contain the intact human *IL3/CSF2* locus. Tracks are shown with (red) and without (black) stimulation of TCR signaling pathways by PMA and calcium ionophore (P/I) for 2 hours [13]. Black arrows represent pDHSs and red arrows are inducible DHSs. Additional previously published H3 K27me3 ChIP-Seq data for murine CD4 naïve T cells and Th2 cells (GEO GSM361998 and GSM362002) [32], and for bulk human CD4 T cells [40] are shown at the bottom of each panel.

example, the inducible transcription factor NF- κ B is recruited more rapidly to the IFN γ promoter after the TCR is stimulated in CD4 memory cells compared to naïve T cells [50].

Once redirected to pDHSs, the TFs bound to these sites are able to stably maintain active chromatin marks such as histone H3 K4me2 (Fig. 2C) and H3 K27Ac (Fig. 3) [13]. Interestingly, these same modifications are typically used in other genome-wide studies to define the locations of conventional enhancers [39], but in our hands, we saw no change in the levels of transcripts for genes that had acquired pDHSs carrying these marks. Consistent with this, it was shown that in resting T cells active chromatin modifications

did not correlate with gene expression but served to poise the genes for rapid induction when the cells were activated [51]. Further, analysis of a chosen subset of pDHSs revealed that they each lacked enhancer activity when tested in conventional transfection assays [13]. Taken together, these data provided further evidence that pDHSs were not functioning as transcriptional enhancers, but specifically as chromatin priming elements.

Several lines of evidence supported the view that pDHSs function primarily to increase local chromatin accessibility at inducible loci: (i) the inducible DHS at the GM-CSF -3 kb enhancer is strongly induced within just 20 minutes in T blast cells, but is undetectable after 4 hours of stimulation in either naïve T cells or thymocytes [13, 52, 53] (Fig. 3B) despite the fact that mRNAs for the TFs which induce the enhancer (NFAT and AP-1) are efficiently expressed in activated naïve T cells [13]. This rapid response corresponds to at least a hundred fold higher rate of NFAT/AP-1-dependent chromatin remodeling in T blast cells; (ii) There is a strong trend for pDHSs to be located within 25 kb of inducible DHSs and inducible genes, and pDHSs typically establish broad active chromatin domains that encompass the closely linked inducible DHSs [13] (Fig. 3B); (iii) The rate of induction of *IL3* mRNA and the DHS at the *IL3* -37 kb enhancer

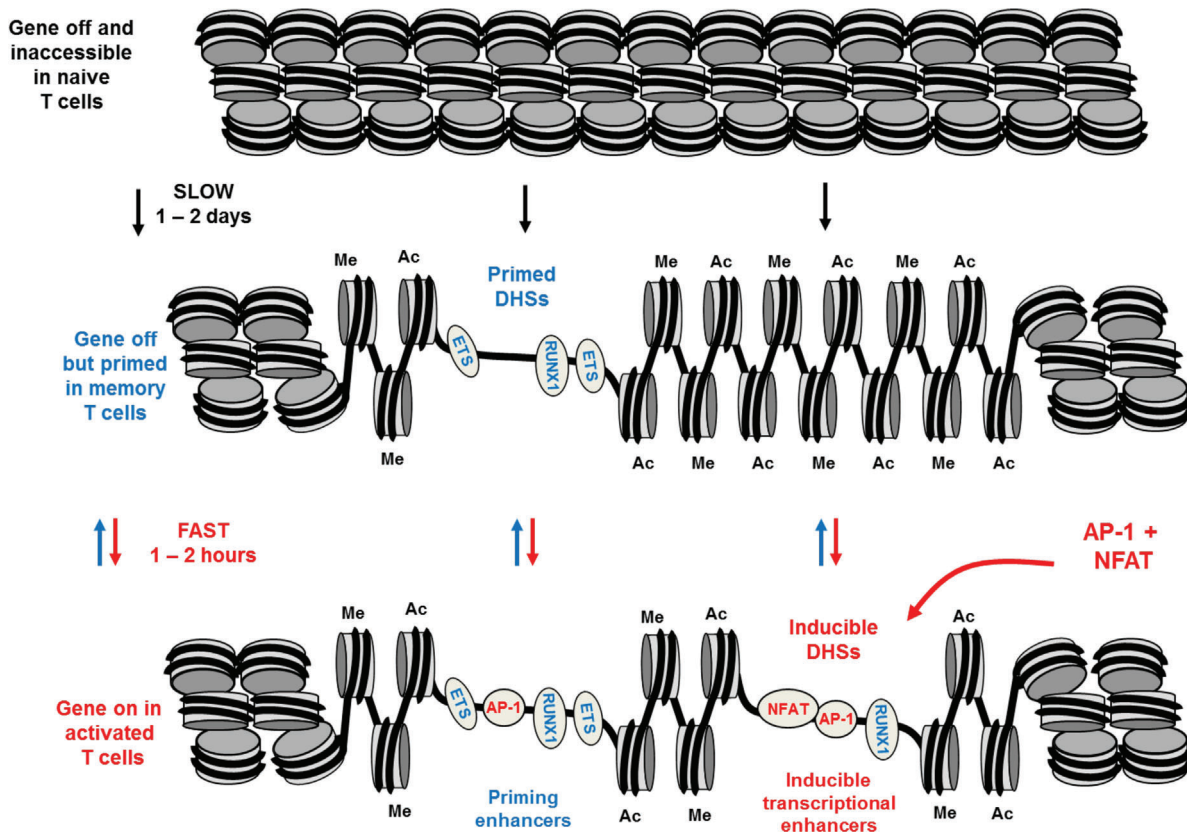


Figure 4. Model depicting mechanisms of acquired immunity in T cells. When naive T cells are activated for the first time they undergo the slow process of blast cell transformation which is driven by transient induction of NFAT and AP-1. As a result of transient AP-1 interactions they also acquire thousands of primed DHSs associated with active chromatin modifications maintained by stable binding of ETS-1 and RUNX1. When T blast cells are re-stimulated they rapidly acquire thousands of inducible DHSs, which are not induced in naive T cells under the same conditions, and many of these correspond to inducible enhancers and promoters activated by NFAT and AP-1. The primed DHSs are effectively functioning as locus priming enhancers, increasing the accessibility of inducible enhancers to NFAT and AP-1. Note that the actual level of chromatin condensation is actually much greater than the stylized views shown here [55], and is likely to be closer in scale to the models depicted below in Fig. 5.

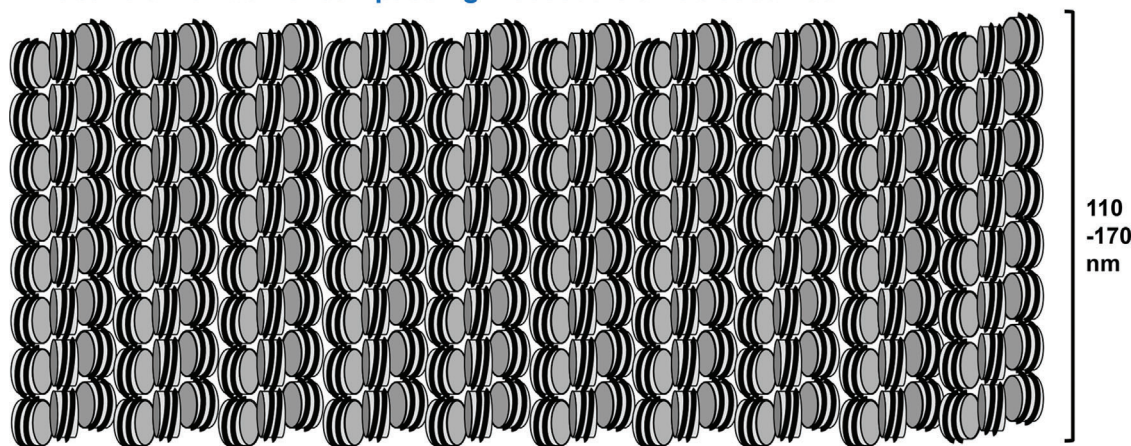
is greatly diminished when a flanking pDHS located at -34 kb is deleted from the genome [13]. What is clear from these observations is that pDHSs act locally to support enhancer function; whereas, enhancers can often act at a great distance from the genes which they activate.

The different kinetics of locus activation described above becomes more comprehensible when one considers that native chromatin normally exists in a highly condensed state. Chromatin becomes more extended as it is activated, but even then it remains highly condensed [54, 55]. Studies of interphase nuclei suggest that most genes are folded at the level of 110–170 nm chromatin fibers [56], which is a level of compaction 10–30 times higher than that of the 30 nm chromatin fiber depicted in most textbooks. Consequently, locus activation is highly dependent on the ability of gene

regulatory elements to recruit chromatin modifying activities that render chromatin more accessible and nucleosomes more mobile. The net effect of these activities is to establish active chromatin domains which are more open and more readily activated. Active chromatin domains may adopt a structure closer to the 30 nm diameter helical structure defined by in vitro studies [57]. Figure 5 loosely depicts what these alternate structures might look like when they are drawn roughly to scale. When viewed in this way it is easy to appreciate that chromatin represents a considerable obstacle to TFs searching for their binding sites on DNA, and that epigenetic modifications to open up the chromatin is likely to be a pre-requisite for efficient locus activation.

The active chromatin domains associated with pDHSs and active genes in T cells are also heavily modified by acetylation of lysines in the histone tails which neutralizes the positive charges on the amino groups which interact with the negatively charged phosphate backbone on DNA (Fig. 3) [13, 34, 38, 58]. These tails are not part of the compact nucleosome core, but extend out from and fold back onto the nucleosomal and linker DNA sequences. The predicted effect of histone acetylation is increased nucleosome mobility resulting from relaxation of the normally tight interactions seen between the histone tails and DNA. Increased nucleosome mobility is also likely to enhance the ability of TFs to access DNA binding sites occupied by nucleosomes, because these nucleosomes need to be displaced by remodelers recruited via TFs. The true nature of the level of folding of active chromatin domains remains unclear, but it is likely that it remains at least as condensed as a 30 nm fiber (roughly as depicted in Fig. 5), and perhaps higher. For example,

Inactive chromatin encompassing inaccessible nucleosomes



Active chromatin domain with accessible mobile nucleosomes and a DHS

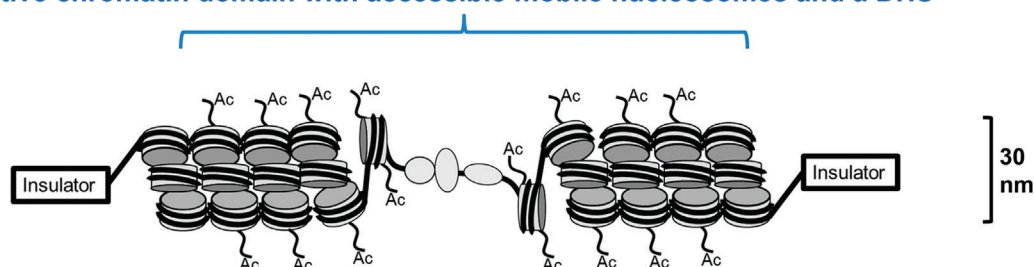


Figure 5. Model depicting chromatin structure transitions when silent loci switch to active chromatin domains encompassing DHSs that maintain the active chromatin modifications that make the chromatin more accessible. These two models are essentially drawn to scale, but are not intended to depict an accurate representation of the nucleosomal organization within these chromatin domains. These models depict chromatin at the level of a 120 nm diameter chromatin fiber (top), which may be typical of inactive loci, and at the level of a 30 nm fiber (bottom) which is essentially the lowest level of folding that might exist inside the nucleus for highly active loci. The borders of the active domains are in at least some cases defined by CTCF-dependent insulator elements which can block the spreading on inactive chromatin domains [80].

the DHS at the GM-CSF enhancer is induced very rapidly in T blast cells even though it remains organized within a highly regular array of positioned nucleosomes which is consistent with a condensed 30 nm chromatin fiber [53]. It is only after induction of this DHS that the local chromatin domain becomes highly disordered as a result of the nucleosomes becoming highly mobile in response to enhancer activation [53].

A flip-flop switch that may partly account for the rapid on-off rates regulating inducible gene expression

Our analyzes of stimulated T blast cells identified several thousand strongly induced DHSs, including ~1,000 DHSs

induced by greater than ten fold, that were associated with inducible genes (Fig. 6A) [13]. However, one currently unexplained finding from these studies is the parallel observation that over 1,000 DHSs are suppressed by TCR signaling by a factor of at least four fold in T blast cells [13]. Many of these diminished DHSs (dDHSs), like the pDHSs described above, are associated with ETS and RUNX motifs, but in contrast, they lack AP-1 motifs. In the *Ccl1* locus, a dDHS is located within a few kb of an inducible DHS at the promoter which encompasses NFAT and NF- κ B-like motifs, and 11 kb from an inducible DHS which recruits the AP-1 protein JUNB [13] (Fig. 6B). This raises the interesting possibility that at least some dDHSs are acting as a sink for nucleosomes which are sliding sideways in response to AP-1, NFAT, and NF- κ B dependent remodeling of the inducible DHSs. Evidence of this effect is provided by the fact that the histone H3 K27Ac- and H3 K4me2-modified nucleosomes flanking these DHSs move closer together in response to stimulation [13] (Fig. 6A and B). This in turn may also provide a mechanism for the subsequent down-regulation of enhancers located at inducible DHSs. In this model, TFs will only re-associate with dDHSs if they can out-compete the chromatin remodeling activities of TFs bound to the adjacent enhancer. The returning TFs could then drive nucleosomes back again to re-occupy the inducible DHSs once levels of NFAT and AP-1 return to levels below a critical threshold. The model depicted in Fig. 6C still needs to be experimentally tested, but it may provide a mechanism for (i) ensuring that responses can terminate just as readily as

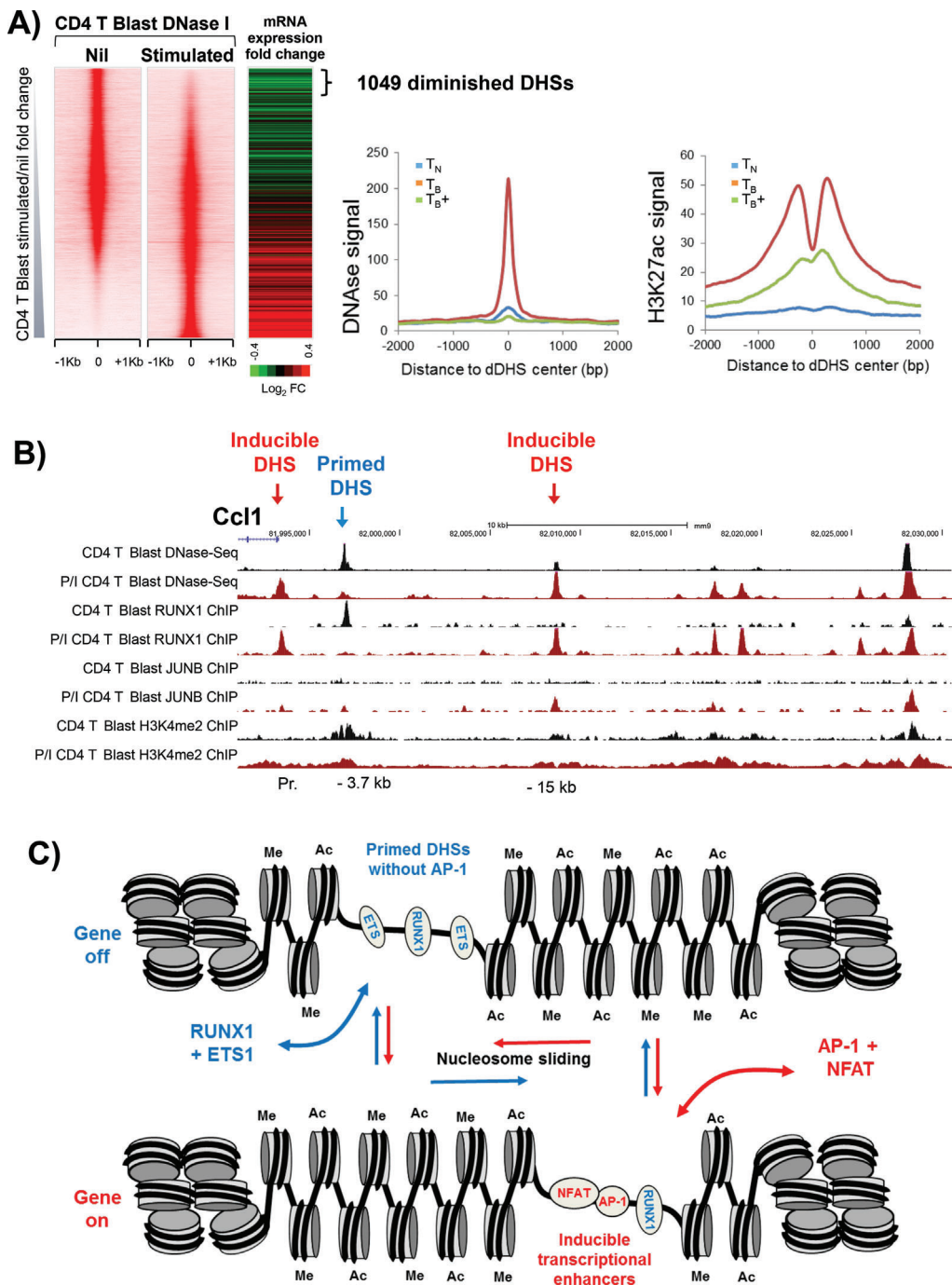


Figure 6. Gene regulation associated with nucleosome sliding. **A:** Genome-wide identification of inducible DHSs enriched in stimulated CD4 T blast cells relative to non-stimulated cells [13]. Profiles are shown for DHSs centered on a 2 kb window and ranked in order of increasing inducibility. Shown alongside are the relative fold changes in mRNA expression for the nearest gene to each DHS, plus the average DHS and H3 K27ac profiles for the diminished DHSs (dDHSs) in naïve T cells (T_N) T blast cells (T_B) and stimulated T blast cells (T_B⁺). **B:** Chromatin structure data for the *Ccl1* locus showing transitions that occur in previously activated CD4 T blast cells during a cycle of activation [13]. A primed DHS located 3.7 kb upstream of *Ccl1* is bound by RUNX1 and flanked by nucleosomes marked by histone H3 K4me2. Following activation, this DHS and binding of RUNX1 are lost, and the gap between the modified nucleosomes disappears as the nucleosomes slide together. In parallel, a DHS bound by RUNX1 is induced at the *Ccl1* promoter and the entire region from the gene to the −3.7 kb DHS is modified by H3 K4me2. The −3.7 kb DHS lacks conventional enhancer activity and is representative of ~1,000 DHSs which are suppressed by TCR signaling [13]. **C:** Hypothetical model based on the above data depicting a flip-flop mechanism whereby nucleosome sliding mediates transitions from the primed to the induced state. In this model it is proposed that primed DHSs that do not interact with AP-1 are unable to compete with DHSs bound by AP-1 and NFAT in terms of excluding nucleosomes following recruitment of chromatin modifiers which mobilize nucleosomes. In this model, the primed DHSs may serve as a reservoir for nucleosomes which are then forced back over the inducible DHSs to end the cycle of activation once levels of NFAT and AP-1 are below a certain threshold.

they are initiated, and (ii) preventing low level chronic signaling from extending the duration of immune responses. This mechanism will suppress low-level chronic activation of a pro-inflammatory response at the point in time when the response needs to be resolved and shut down again. This represents an alternate means of shutting down regulatory elements which otherwise can remain active and nucleosome-free until the activating factors are depleted [59]. This process of nucleosome repositioning is reminiscent of the recently described process of “assisted loading” whereby transient binding of TFs can direct nucleosome repositioning in a hit-and-run mechanism to assist the binding of a second TF [60].

Although we can speculate about a functional role for some pDHSs at genes such as *Ccl1*, the role of most of the pDHSs defined in Fig. 6A remains obscure. Many dDHSs are located far from genes and other DHSs, and Fig. 6A suggests that the suppression of pDHSs is more often associated with down-regulation and not activation of gene expression. Consequently, the majority of pDHSs are likely to reflect genes that are simply repressed by TCR activation.

The need for new definitions of enhancer elements

Currently, the widely adopted convention for genome-wide studies is to refer to all DHSs carrying specific active histone modifications as enhancers [39]. Furthermore, the latest trend is to refer to complex clusters of DHSs and promoters as “super-enhancers” [61]. However, these trends overlook the complexities of gene regulation and make no allowance for different classes of DHSs having distinct functions while cooperating in the activation of transcription. One recent study highlighted the deficiencies of the widely adopted current approaches of using H3 K4me and H3 K27Ac to define enhancers by showing that just 11% of 4,000 such regions had strong enhancer assays in classical enhancer assays [62]. Similar findings came from another study which found histone modifications alone were insufficient to reliably identify enhancer elements [63]. Our recent study has now helped resolve some of the confusion surrounding this field by showing that different classes of DHSs marked by H3 K4me2 and H3 K27Ac can have very different functions. We also found that many of these pDHSs lacked classical enhancer activity measurable in transfection assays, and in their natural context they clearly did not function as enhancers to activate steady-state transcription [13]. The role of these elements appeared to be strictly to increase the kinetics of gene activation by making nearby inducible enhancers and promoters more accessible to the inducible TFs that activate them. This distinct class of pDHSs, while they superficially resemble true transcriptional enhancers, should perhaps be referred to as either “chromatin priming elements” or “priming enhancers,” so as to make a distinction.

It is now becoming clear that many other cell types and TFs are also likely to regulate transcription via similar interactions between different classes of regulatory elements, whereby a priming phase precedes the transcription

activation phase. The original definition of the “Locus Control Region” (LCR) was based on a complex cluster of four DHSs in the β globin locus which cooperated to support correct developmentally regulated gene expression [64–66]. However, these DHSs varied widely in their capacity to activate transcription, suggesting that these elements are not all functioning in the same way. The term “super-enhancers” has been used to define large active chromatin domains encompassing multiple DHSs (including promoters) [39], but this fails to take into account the complex interactions at work between all these different elements, as exemplified by the alpha globin locus [67]. This term is at best an acknowledgement of the complex nature of what lies underneath. Clearly, there is a pressing need for the gene regulation community to adopt more informative definitions for gene regulatory elements which are currently being lumped together as similar entities. Chromatin priming elements are one such entity needing a new definition, as part of larger entities that include clusters of DHSs experimentally defined as LCRs. It may eventually become commonplace for LCRs to be found to consist of a mixture of priming enhancers and transcriptional enhancers that function at different stages of development or in different environments.

Parallel mechanisms cooperate to boost immune responses in T cells

Chromatin priming elements now represent an additional layer of regulation among the many mechanisms at work which boost the activation of immune responses in T cells. At the level of epigenetics, the priming elements are adding another layer of chromatin regulation to a subset of genes which are repressed by Pc-dependent mechanisms in naïve T cells [28]. At the post-transcriptional level, it is clear that many inducible mRNAs have short half-lives and are stabilized in activated T cells, leading to higher protein levels and a longer duration of responses [68]. At the level of inducible TFs it is known that higher levels of NFAT proteins are expressed in activated T cells compared to naïve T cells [69], in contrast to the more modest changes seen at the mRNA level [13]. Previously activated T cells also have enhanced proliferative responses due to higher levels of expression of the receptor for the T cell growth factor IL-2 [70, 71].

Conclusions and prospects

The field of eukaryote gene regulation has made great strides in the last 35 years since the first characterizations of promoter elements, the discoveries of enhancers and TFs, and observations that active genes lie within extensive domains of accessible chromatin [55]. Since then, the known mechanisms controlling locus activation are becoming increasingly complex, but it is still accepted that active genes lie within active chromatin domains established by interacting cis-regulatory elements, which in some cases are defined as LCRs [72, 73]. Therefore, we need to further refine

our definitions of the DNA elements that control patterns of developmentally regulated tissue-specific and inducible gene expression. We also need to determine how patterns of gene regulation are stably maintained and re-established after mitosis.

To gain a fuller understanding of the basis of immunological memory there are several areas requiring closer inspection: (i) A hit-and-run mechanism of epigenetic priming needs to be mitotically stable in rapidly dividing T blast cells. In other model systems it has been observed that RUNX and GATA family TFs can function as mitotic bookmarks [74, 75]. Given that pDHSs encompass composite RUNX/ETS motifs, which are likely to form relatively stable complexes, they also represent good candidates for regulatory elements functioning to preserve patterns of gene expression in dividing cells. (ii) Immunological memory can be maintained for decades, and is known to be dependent on cytokines such as IL-7 and TNF superfamily receptors such as OX40 [76–78]. Given that these pathways are also linked to AP-1 it is likely that there is some requirement for intermittent low level activation of AP-1 in order to reinforce imprinting of the epigenetic program at pDHSs. This is probably why immunological memory is so stable even in the prolonged absence of TCR signaling. (iii) Many immune response genes show polarized patterns of gene expression following differentiation to different classes of T cells such as Th1 and Th2 cells. However, loci such as IL-4 and IFN γ show the first signs of activation in the form of pDHSs which are gained prior to terminal differentiation [13]. Because a subset of pDHSs are also enriched for GATA3 and TBX21 binding motifs, it is likely that they have the additional role of making target loci receptive to the alternate differentiation inducing signals that they might subsequently encounter. Taken together with their other known properties, all of these features allow priming at pDHSs to play a vital central role in the establishment and maintenance of immunological memory and in ensuring appropriate responses at immune response genes whereby they are normally maintained in a silent state but can be rapidly reactivated when required.

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